

**Immunohistochemical identification of mismatch repair
gene deficit and its clinico-pathologic significance in
young patients with colorectal cancer**

Muhammad Fayyaz Hameed

MBBS (Punjab), FCS (SA)

A research report submitted to the Faculty of Health Sciences, University of Cape Town, in partial fulfilment of the requirements for the Degree of Master of Medicine in the branch of
General Surgery.

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DECLARATION

I, Muhammad Fayyaz Hameed, declare that this research report is my own work. It is being submitted as partial fulfilment for the degree of Master of Medicine, in the branch of General Surgery, at the University of Cape town, South Africa. It has not been submitted before for any degree or examination.

Signed by candidate

Dr. M F Hameed

November 2005

Congress Presentations arising from this work

1. Congress of the Surgical Research Society of South Africa – July 2000, Cape Town, South Africa.

The use of Immunohistochemical Detection of Mismatch Repair Deficit in Patients with Colorectal Cancer. MF Hameed¹, P Hall², R Barnard², U Algar¹, R Felix³, RS Ramesar³, PA Goldberg¹. Department of Surgery¹, Anatomical Pathology² and Human Genetics³, Groote Schuur Hospital and University of Cape Town, South Africa

2. South African Gastroenterology Society Congress – August 2001, Durban, South Africa.

The role of Immunohistochemistry in the Identification of Mismatch Repair Gene Deficit in Young Patients with Colorectal Cancer. MF Hameed¹, P De La M Hall², R Barnard², RS Ramesar³, PA Goldberg¹. Department of Surgery¹, Anatomical Pathology² and Human Genetics³, Groote Schuur Hospital and University of Cape Town, South Africa

(See Appendix A & B)

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List of Abbreviations

APC	Adenomatous polyposis coli
DNA	Deoxyribonucleic acid
FAP	Familial adenomatous polyposis
GSK	Glycogen synthase kinase
HNPCC	Hereditary nonpolyposis colorectal cancer
H&E	Haematoxylin and eosin
LOH	Loss of heterozygosity
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	High frequency microsatellite instability
MSI-L	Low frequency microsatellite instability
PCR	Polymerase chain reaction

ABSTRACT

An immunohistochemical technique is used in this study to detect mismatch repair deficit in young patients with colorectal cancers. Ninety-three patients who were 45 years of age or younger at the time of diagnosis of colorectal cancer were studied. Paraffin sections of tumour and adjacent normal colonic tissue were dewaxed and hydrated. After unmasking antigenic sites, the monoclonal hMLH1 and hMSH2 antibodies were applied in a dilution of 1/70 and 1/100 respectively in a solution of 0.2x casein in Tris buffered saline (pH 7.6) at room temperature for 2 hours. Two investigators separately assessed the results in coded slides. The presence of mutation in the genes was shown by absence of staining in tumour cell nuclei, implying the absence of normal protein product of mismatch repair gene. The clinical, pathological and survival characteristics of the groups with and without mismatch repair defect were compared. The mismatch repair status of these young patients as detected immunohistochemically was then correlated with the results of genetic mutation testing where available. Twenty five (40%) of 93 patients were negative for hMLH1 protein expression and 12/93 negative for hMSH2, indicating abnormal MMR gene protein. These patients with mismatch repair defect had significantly more right sided tumours; these tumours were more often poorly differentiated and significantly more tumours in the mismatch repair defect groups had >50% mucinous component. The survival between the two groups was not statistically significantly different. Of the 56 patients who did not show MMR defect on immunohistochemistry, 27 had mutation analysis done and none of them showed any disease causing mutations in *hMLH1* or *hMSH2*. Immunohistochemistry should be considered as initial investigation in young patients with colorectal cancer to identify patients who have MMR gene defect. This will also lead to the identification of new families with HNPCC.

1. Introduction and Literature Review

Colorectal cancer is a common malignancy in some areas of South Africa. According to South African National Cancer Registry the lifetime risk of developing colorectal cancer in South Africa is 1 in 94 for males and 1 in 130 for females. In USA, comparable life time risk of developing colorectal cancer is 1 in 16.¹ Due to heterogeneity of the South African population and their varied dietary habits this risk is different in different population groups. For example, the lifetime risk of developing colorectal cancer is 1 in 34 for white males but only 1 in 400 for black males.² Between 1993 and 1995, colorectal cancer was second most common cancer in Asian males in South Africa while it ranked 3rd and 10th in white and black males respectively. In females during the same period, colorectal cancer was 2nd most common malignancy in white women (5.2%), 3rd in mixed race population (3.3%), 4th in Asian women (4.9%), and 7th most common malignancy in black women (1.9%). Annual incidence of colon cancer is 23 per 100,000 of population in South African whites while it is 3 per 100,000 in black population. Comparable rates in USA are 31 and 32 respectively. This means that environmental factors are very important in the development of colorectal cancer. It is likely that as the level of affluence increases in previously disadvantaged people, it will lead to increased urbanization and changing lifestyle that will probably result in an increase in the incidence of colorectal cancer in the black South African population.

A proportion of colorectal cancers are hereditary. Among high incidence populations, the proportion of hereditary cancers is low³ but in low incidence populations, the hereditary component is likely to be larger. Two most common forms of hereditary

colorectal cancer are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). These accounts for about 1% and 1-6% of colorectal cancers respectively.⁴⁻⁶ At least another 5-8% of colorectal cancers seem to be familial in nature although the exact hereditary basis of these cases is not yet clearly defined.⁷ Studies on these hereditary forms of colorectal cancer have led to new insights towards the understanding of pathogenesis of colorectal cancer.

1.1 Pathogenesis of colorectal cancer

All cancers result from an accumulation of genetic alterations that leads to an imbalance between cell differentiation and cell proliferation.^{8,9} In case of colonocytes, this results in the expansion of a polyclonal crypt cell population at the base of colonic crypts. This is an early event in the development of colorectal cancer.¹⁰ Colorectal cancer provides a good model for studying genetic alterations involved in carcinogenesis because it is very common and there is a precursor lesion in the form of adenoma that can be easily identified and removed from the colon for cytogenetic analysis. In addition, hereditary forms of colorectal cancer provide invaluable opportunity to study the process of carcinogenesis in patients who are known to have a very high risk of developing colorectal cancer. Initial molecular and cytogenetic studies showed that losses of chromosome 17 (this later became known as *p53*) and 18 sequences occurred frequently in colorectal carcinomas.^{11,12} In 1988, Bert Vogelstein and colleagues studied varying sizes of colonic adenomas and advanced colonic carcinomas for genetic alterations. They confirmed that these genetic alterations were either mutations in oncogenes like *k-ras* or allelic losses on chromosomes 5, 17 and 18.¹³ These alterations were more common in carcinomas and generally less common in adenomas. For instance, allelic deletions of chromosome 18

were present in 11% of early adenomas, 47% of large adenomas and 73% of carcinomas. The authors suggested that colorectal tumour development is a progressive staged process where accumulations of genetic alterations lead to progressively more advanced lesions. Other studies confirmed these findings and showed that loss of heterozygosity (LOH) was a consistent feature in majority of colorectal tumours.¹⁴ Curiously, it was noted that colorectal tumours that do not demonstrate this pattern tend to occur on the right side of the colon.¹⁵ A logical conclusion of these studies was that there must be other mechanisms responsible for the development of some colon cancers in addition to the one that involved mutations in proto-oncogenes and deletions of tumour suppressor genes. A mechanism responsible for the majority of these unexplained tumours was discovered in 1993 and has become known as the mismatch repair mechanism, microsatellite instability pathway or microsatellite mutator pathway.¹⁶⁻¹⁸ Defects in this mechanism underlie the development of carcinoma in hereditary nonpolyposis colorectal cancer. A great majority of colorectal cancers arise from either the chromosomal instability or microsatellite instability (MSI) pathway.

1.1.1 Chromosomal instability pathway

Vogelstein and colleagues proposed this mechanism of development of colorectal cancer.¹³ Alternative names for this pathway are the adenoma-carcinoma sequence or the adenomatous polyposis coli (APC)/ β -Catenin pathway.¹⁹ This pathway accounts for most sporadic colorectal cancers and is responsible for development of colorectal cancer in patients with familial adenomatous polyposis. The most important gene involved in this pathway is the tumour suppressor *APC* gene located on chromosome 5q. Germline mutations in this gene result in Familial Adenomatous Polyposis.^{20,21}

The *APC* gene mutation is an early event in the pathogenesis of sporadic colorectal cancer via chromosomal instability pathway. Subsequent deletion or mutation of the wild type allele results in loss of tumour suppressor function and uncontrolled growth and proliferation of colonic cells at the base of the colonic crypts.²² This leads to the formation of early adenoma.

During the adenomatous stage mutations in the proto-oncogene *k-ras*, the cell cycle regulator gene *p53* (previously known as deletion on chromosome 17) and deletions on chromosome 18q result in progression to invasive malignancy.¹³ Colorectal cancers developed through this pathway exhibit loss of heterozygosity, chromosomal amplifications, translocations and aneuploidy.⁹

APC gene product mediates its functions by binding to many intracellular proteins including β -catenin, γ -catenin, glycogen synthase kinase (GSK)-3 β , axin, tubulin and others.¹⁰ These proteins in turn regulate cell proliferation and apoptosis. Mutations in *APC* result in dysregulation of β -catenin and other proteins by disruption of the β -catenin/APC/axin/GSK-3 β complex. As a result, β -catenin is released from the complex, migrates to the nucleus and results in abnormal gene expression through interaction with T-cell factor/lymphoid enhancer factor. The target genes activated by T-cell factor/ β -catenin are thought to be responsible for uncontrolled cell proliferation.^{19,23} This pathway of colorectal carcinogenesis is shown in a simplified manner in Figure 1.

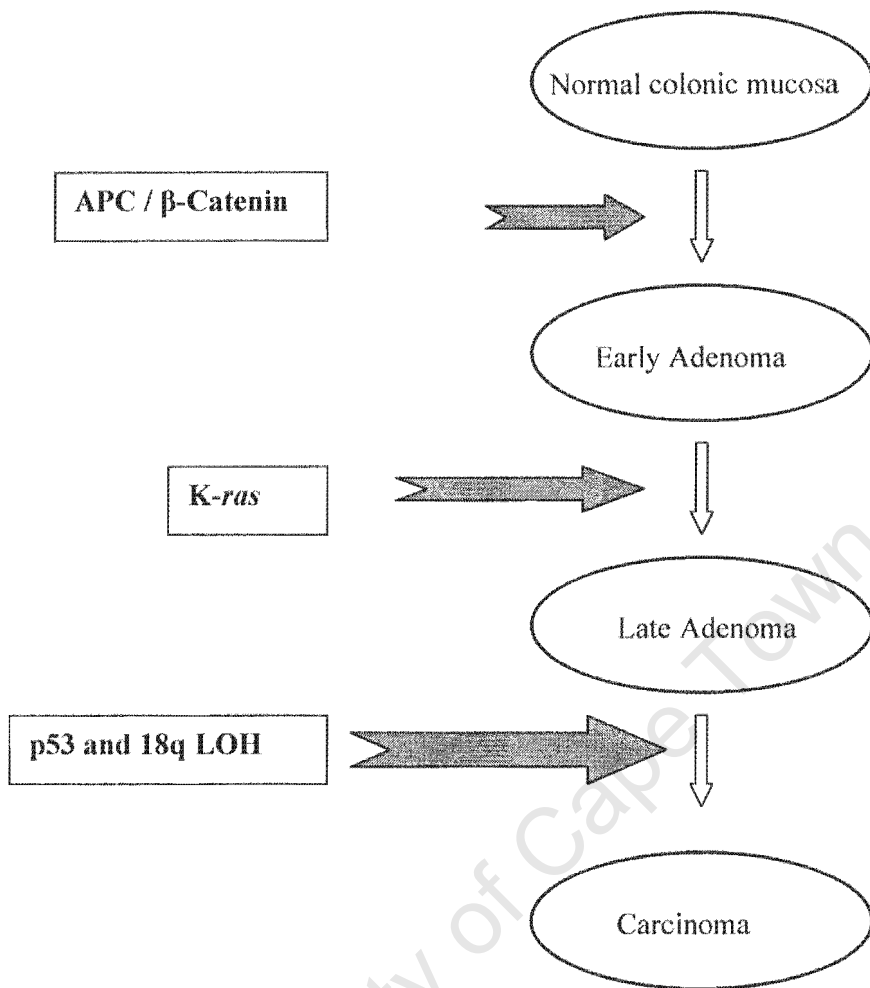


Figure 1: Progressive staged developed of colorectal cancer through chromosomal instability pathway (APC/ β -Catenin pathway). Only the critical genes involved in the process of carcinogenesis are shown in this figure. (Adapted and modified from Reference 17).

1.1.2 Microsatellite instability (MSI) pathway or the DNA mismatch repair (MMR) pathway

Early studies on the pathogenesis of colorectal cancer showed that colorectal tumours that did not show loss of heterozygosity, aneuploidy, and other chromosomal instabilities were more often located in the proximal colon.¹⁵ The genetic basis of this different phenotype, which was similar to HNPCC, was initially not clear. Molecular analysis of tumours from patients with hereditary nonpolyposis colorectal cancer syndrome and linkage analysis of two large HNPCC kindreds showed that the gene responsible for this syndrome was probably located on chromosome 2. The prevailing hypothesis at that time was that most loci coding tumour suppressor genes (for example *APC*) are deleted during carcinogenesis. In contrast both alleles of chromosome 2p were retained in these HNPCC tumours.^{17,24} This was an unexpected finding and meant that the gene responsible for HNPCC tumours was not a tumour suppressor gene. Another unexpected finding of the same study was somatic alterations of numerous microsatellite sequences in HNPCC tumours. Ionov *et al.*, while studying genetic alterations in sporadic colorectal tumours found deletions in mono-, di- and tri-nucleotide repeats (also known as microsatellites) in 16% of colorectal cancers.¹⁶ These cancers were mostly right sided and poorly differentiated. The incidence of *k-ras* and *p-53* mutations in these tumours was much lower compared with the ones without deletions in microsatellites. It was suggested that these mutations in microsatellites were so widespread and ubiquitous that they were most probably due to loss of fidelity of the replication machinery of cells. Similar findings of loss or insertion of mono-, di- or tri-nucleotide repeats in microsatellite sequences were reported in other studies of sporadic colorectal cancers.¹⁸

Interestingly these sporadic tumours had some features characteristic of HNPCC tumours such as right-sided location, poor differentiation, and near diploidy. These data suggested that HNPCC and a subset of sporadic colorectal tumours were associated with a similar genetic defect resulting in replication errors in microsatellites. The defect was a germline mutation in patients with HNPCC and a somatic mutation in apparently sporadic colorectal cancers. These replication errors in microsatellites were already known to occur in lower animals like *Escherichia coli* and yeast. It was also known that these replication errors resulted from mutations in replication error repair genes or mismatch repair genes in these organisms.^{25,26,26} Soon the search for similar genes in human genome started and two genes responsible for maintaining replication fidelity or mismatch repair were identified.²⁷⁻³⁰ These genes were named *hMSH2* and *hMLH1* and they were located on chromosome 2p and 3p respectively. Mutations in these genes have been identified in HNPCC families and these mutations represent about 90% of all mutations identified in these cases. Three more genes, *hMSH6*, *hPMS1*, and *hPMS2* have been identified that are involved in mismatch repair but mutations in these genes represent a minority of patients with HNPCC.^{7,31}

How does a mismatch repair deficit and resulting insertions or deletions in microsatellite sequences result in carcinogenesis? Microsatellites are tandem repeat sequences of mono-, di- or trinucleotides abundantly present throughout the human genome. They show polymorphisms and are often used as markers for certain genes in linkage analysis. Most of these microsatellites are in non-coding regions of the human genome and so a change in length in these locations is not expected to have any significant effect. However, microsatellites also occur in coding exons of certain

genes e.g. type II transforming growth factor- β receptor gene and *BAX* gene (an apoptosis regulator).³²⁻³⁴ With mutations in microsatellite sequences in coding exons of these genes, the protein products of these genes are absent or non-functional. Since these genes are involved in cell proliferation and apoptosis regulation, their mutations lead to an imbalance between cell proliferation, differentiation and apoptosis that results in carcinogenesis. The final molecular mechanisms underlying these changes are yet unknown. A mismatch repair gene defect also increases the rate of mutations in the microsatellite DNA by many thousand folds compared to the rate of random mutations occurring in the DNA in the presence of a functional mismatch repair machinery.¹⁶ This leads to increased probability of mutations occurring in the microsatellite sequences of coding regions of the genes mentioned above.

Majority of sporadic colorectal cancers arising due to MMR gene defect display inactivation of *hMLH1*. Mutations in other genes like *hMSH2*, *hPMS1* and *hPMS2* are not common in this subset of colorectal cancers. The most common reason for inactivation of the *hMLH1* in these cases is hypermethylation of the promoter region of the gene.^{35,36} This results in a mismatch repair defect and increased rate of mutations in microsatellites throughout the genome.

Microsatellite instability (MSI) is a molecular phenomenon. It is hallmark of mismatch repair deficit. MSI has been divided into high- and low- frequency microsatellite instability depending on the number of microsatellite loci showing instability in a particular tumour. The detection of microsatellite instability is a relatively expensive process and requires staff intensive microdissection of the tumour to retrieve tumour DNA. The normal DNA for comparison with tumour DNA

is usually extracted from blood sample of the patient. Then both normal and tumour DNA are subjected to polymerase chain reaction (PCR) based assay and the number of microsatellite markers showing instability in the tumour DNA compared with normal DNA of the same subject is analysed. This needs significant technical expertise that is not widely available in South Africa. In the United States, the National Cancer Institute has recently published guidelines for determination of MSI in a standardized fashion. According to these guidelines, MSI has been divided into 2 subgroups:

- 1) High frequency of microsatellite instability (MSI-H) if instability is found at \geq 30% of the loci studied
- 2) Low frequency of microsatellite instability (MSI-L) which means instability at 1% to 29% of loci. Uncertainty exists about the exact clinical and biological significance of MSI-L.^{37,38}

1.2 Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

Nearly a hundred years ago Aldred Warthin, a pathologist, studied a family and published his observations in 1913.³⁹ He found that this family had multiple cancers in various organs over many generations and he suggested that the cancer disposition was inherited. Lynch and Krush restudied this family and called it family G. The condition became known as Lynch syndrome.⁴⁰ The genetic and molecular basis of its pathogenesis was elucidated in 1993.¹⁷ The term HNPCC was adopted in late eighties.

HNPCC is the most common form of hereditary colorectal cancer accounting for 1-6% of colorectal cancers. It is autosomal dominant and results from germline mutations in one or more of at least five mismatch repair genes located on chromosomes 2, 3 and 7. Mutations in *hMLH1* and *hMSH2* account for the vast majority (90%) of patients with HNPCC while a minority have mutations in *hMSH6*, *hPMS1* and *hPMS2*.⁴¹

HNPCC syndrome results in colorectal neoplasia in multiple generations. It usually presents at an early age with the average age of diagnosis being approximately 45 years).⁷ Most of the tumours are located proximal to splenic flexure. There is an excess of synchronous and metachronous colorectal cancer. The extracolonic cancers associated with this syndrome are carcinoma of the endometrium, ovary, stomach (particularly in Asian countries such as Japan and Korea), small bowel, pancreas, hepatobiliary tract, brain and upper uroepithelial tract.^{7,42-45} For clinical identification of these families and for the purpose of uniformity in collaborative studies across the globe, a set of criteria for identification of families with HNPCC was established in 1990.⁴⁶ This became known as Amsterdam criteria. The criteria were later found to be too restrictive and have been recently revised.⁴⁵ According to the new Amsterdam II criteria, an HNPCC family is defined as follows:

- At least three relatives must have a cancer associated with hereditary non-polyposis colorectal cancer (colorectal, endometrial, stomach, ovary, ureter or renal-pelvis, brain, small bowel, hepatobiliary tract, or skin [sebaceous tumours])
 - One must be a first-degree relative of the other two
 - At least two successive generations must be affected

- At least one of the relatives with cancer associated with hereditary nonpolyposis colorectal cancer should have received the diagnosis before the age of 50 years.
- Familial adenomatous polyposis should have been excluded in any relative with colorectal cancer.
- Tumours should be verified pathologically whenever possible.

In South Africa, HNPCC has been identified in some large families in the North-Western Cape. Mutations have been identified in both *hMLH1*,⁴⁷ and *hMSH2* genes in these families.

HNPCC is probably under-reported in South Africa. This may be due to

- 1) Lack of awareness on the part of the clinicians
- 2) Physicians not making a concerted effort to get proper family history.
- 3) Scarcity of financial and human resources to perform MSI testing on resected specimens.
- 4) Lack of public awareness of the hereditary nature of the problem
- 5) Limitation of mutation testing to mutations in *hMLH1* and *hMSH2* genes only until recently.

Human Genetics department of University of Cape Town has recently started looking at mutations in *hMSH6*.

1.3 Colorectal cancer in young patients

A greater proportion of young patients who develop colorectal cancer are likely to have a mismatch repair defect than cancers arising in older patients.^{48,49} In addition

HNPCC can be detected in up to 15% of young patients presenting with what initially appears to be sporadic colorectal cancer.⁵⁰ Many of the clinical and morphological characteristics of sporadic colorectal cancers in young patients are similar to those of HNPCC associated tumours. Such patients need to be screened for this inherited defect. Due to younger age and increased risk of metachronous tumours, therapeutic options might be different in younger patients than their older counterparts.

1.4 Significance of determination of mismatch repair deficit

The presence or absence of mismatch repair defect has far reaching implications in the management of young patients with colorectal cancer. If a patient has a mismatch repair defect, he or she might be carrying a germline mutation and belong to an HNPCC family. If so, then the patient might need subtotal colectomy rather than segmental colectomy for a colonic tumour because of the increased risk of metachronous cancers. The family of such patients will need to be counselled and screened for the presence of mutation detection and those members of the family positive for a mutation will need surveillance for detection of early colorectal cancer and extracolonic cancers.

There is evidence that tumours with high-frequency MSI (i.e. having mismatch repair deficit) respond differently to chemotherapy compared to those that do not show high frequency MSI.^{51,52} Detection of mismatch repair deficit may lead to prediction of response to a specific type of chemotherapy.⁵³ Patients with stage II or III colon cancer⁵⁴ whose tumours exhibit high frequency microsatellite instability do not benefit from fluorouracil based adjuvant chemotherapy.⁵⁵ Cancers arising as a result

of mismatch repair defect may have better survival than those arising through chromosomal instability pathway. This effect is more apparent in Duke's C cancers.⁵⁶

Consequently, it is important to know the mismatch repair status of a colorectal cancer patient, if possible before starting therapy. But the determination of mismatch repair status needs testing for microsatellite instability which unfortunately is expensive, staff intensive and needs specific expertise not widely available in South Africa. Recently an immunohistochemical test has been described to detect mutations in *hMLH1* and *hMSH2* genes by detecting the presence or absence of their protein end products.^{57,58} This test can be performed on formalin fixed, paraffin wax embedded tumour tissue. The test is simple, rapid and relatively inexpensive compared to PCR based microsatellite instability assays. It can be readily learnt by anybody proficient in immunohistochemical techniques.³⁷

At Groote Schuur Hospital Cape Town, the Colorectal Surgery Unit is actively involved in the management of patients and families with HNPCC in collaboration with Departments of Anatomical Pathology and Human Genetics of the University of Cape Town. The unit also has a particular interest in the management of colorectal cancer in young patients because of the high frequency of cancers in this population due to HNPCC. Until now mutation testing has been done for all patients suspected of having HNPCC related colorectal cancer. But this procedure is expensive and time-consuming. If immunohistochemistry can reliably identify colorectal cancer patients with a mismatch repair gene defect, then only those patients could be subjected to mutation analysis. The study reported in this thesis describes the results of

immunohistochemical identification of mismatch repair gene deficit and its clinicopathologic significance in young patients with colorectal cancer.

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2. Aims of the Study

This is a retrospective study. The aims of the study are the following:

1. To determine the mismatch repair deficit status by immunohistochemistry in patients 45 years of age or younger at the time of diagnosis of colorectal cancer.
2. To compare the clinical and pathological features of colorectal tumours with mismatch repair gene defect to those without this defect in patients aged 45 years or younger
3. To correlate the results of immunohistochemical detection of mismatch repair defect with results of genetic mutation testing where available

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4. Patients and Methods

3.1 Data collection and specimen retrieval

Records of all patients from January 1983 to December 2001 who had colorectal cancer diagnosed at the age of 45 years or younger at Groote Schuur Hospital were reviewed. The histopathology reports of their diagnosis were retrieved from hospital records and from the archives of the Division of Anatomical Pathology to confirm the diagnosis of colorectal cancer. Only those patients who had histological slides and blocks archived in the Division of Anatomical Pathology Laboratory at Groote Schuur Hospital/University of Cape Town were included in the study.

- The demographic and clinical data relating to the colorectal cancer were collected using a standardized data collection form. The data from the hospital record for each patient were then entered into a database programme (Microsoft Access).
- Follow up data for survival analysis were collected from patients' hospital records, family colon cancer management office and by telephone if necessary. For the purpose of this study, patients who were alive and their follow up was up to date were censored on 30th June 2002.
- The histopathological data were partly recorded from the original histopathology report. This data were confirmed during initial examination of the original haematoxylin and eosin (H&E) slides by a pathologist and

information missing in the pathology report was recorded in the database along with the demographic and clinical data.

- Slides showing both the tumour and normal colonic epithelium were selected and corresponding paraffin embedded tissue blocks were retrieved for sectioning to prepare for immunohistochemical staining.
- The slides were coded before immunohistochemical staining so that when the immunohistochemically stained slides were reviewed, the demographic, clinical and morphological information about the patient and the tumour was not available to the reviewing investigators.
- The mismatch repair status of the tumour was detected by the immunohistochemical technique described below by the presence or absence of expression of the protein end product of *hMLH1* and *hMSH2*.
- Two slides were used for each patient. One for anti-hMLH1 and the other for anti-hMSH2 staining. In some cases normal tissue could not be seen on a slide as it only contained tumour tissue. In these cases separate sections were cut from blocks of normal colonic tissue for anti-hMLH1 and anti-hMSH2 staining to serve as positive controls.
- In cases of synchronous lesions (nine cases), only one tumour randomly selected was sectioned and stained

- In metachronous lesions (two cases), second tumours were sectioned and stained. The original cancer was resected but the slides and blocks were not available for the study.
- When the immunohistochemical staining was complete and the coded slides were reviewed, each tumour was examined for presence or absence of expression of *hMLH1* or *hMSH2* gene protein. For the purpose of this study if >50% of the tumour in the section examined stained with either anti-*hMLH1* or anti-*hMSH2* antibody, this was considered positive staining. This meant that the tumour expressed the corresponding MMR protein and there was no MMR defect.

3.2 Immunohistochemical staining

A pilot study was performed before doing the final immunohistochemical staining and a variety of antigen retrieval methods were tried. Antibodies were used in different dilutions to establish an optimum method of staining. Optimum results were obtained using heat mediated antigen retrieval (HMAR) and using antibodies at a dilution of 1/70 for anti-*hMLH1* and 1/100 for anti-*hMSH2* antibody. Sporadic colon cancers from patients without any family history of cancer, and tumours from known HNPCC patients were stained with both anti-*hMLH1* and anti-*hMSH2* antibodies and results were evaluated in a “blinded” fashion. The results of this pilot study showed that the technique as described below works in our setting (raw data not shown). Then tumours of patients included in the present study were stained with both anti-*hMLH1* and anti-

hMSH2 antibodies. The results of the pilot study were presented at Surgical Research Society of South Africa meeting in 2000. The abstract is included as appendix A.

Formalin fixed paraffin processed blocks of colorectal cancers in patients' ≤ 45 years of age were sectioned and mounted on APES coated slides. After dewaxing and hydrating, endogenous peroxidase activity was quenched using 3% (aq.) hydrogen peroxide (H_2O_2) solution. Antigenic sites were unmasked by heat-mediated antigen retrieval (HMAR) for 2 minutes at full pressure in antigen unmasking solution (Vector Laboratories) by means of a Presto pressure cooker. The monoclonal hMLH1 antibody, clone G168-15 (Pharmingen, San Diego, California, USA) was applied at a final dilution of 1/70 in a solution of 0.2x casein in Tris buffered Saline (pH 7.6) at room temperature for 2 hours. The monoclonal hMSH2 antibody, Clone FE11 (Catalogue number NA27, Oncogene Research Products, Cambridge, MA) was applied at a final dilution of 1/100 in a solution of 0.2x casein in Tris buffered saline (pH 7.6). A streptoavidin-biotin complex / HRP duet kit K0492 (Dako A/S Copenhagen) was applied as per instructions. Positivity was developed by applying the chromogenic substrate, diaminobenzidine (DAB) (K3466, Dako, A/S Copenhagen). Sections were lightly counterstained in haematoxylin, blued in Scotts tap water substitute, dehydrated through graded ethanol, to xylol, and mounted in a synthetic resin.

Normal colonic mucosa adjacent to the tumour served as positive control which expressed the protein end product of *hMLH1* and *hMSH2*. Where no normal colonic tissue was present in the section, separate sections were cut from the blocks of normal colonic tissue from the same patient as the tumour and stained to serve as positive controls.

3.3 Statistical analysis

All statistical analysis was done using the software *Statistica 6*.⁵⁹ Categorical variables were compared with chi-square test and Fisher's Exact Test. Survival curves were developed using Kaplan-Meier method and survival in the two groups was compared using log-rank test. A p value of less than 0.05 was considered significant for the purpose of this study.

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4. Results

In normal colonic tissue or a tumour without mismatch repair defect, hMLH1 and hMSH2 proteins are expressed mainly in the nuclei but sometimes a blush is also noted in the cytoplasm when immunohistochemical staining is performed. The monoclonal antibody to hMLH1 and hMSH2 binds to this protein and shows positive staining. On the other hand in a case with colorectal cancer due to mismatch repair defect, one of the hMLH1 or *hMSH2* proteins is not expressed and there is no staining in the nuclei or cytoplasm in tumour but the surrounding normal colonic epithelium shows staining. Figure 2 is an example of colonic cancer with adjacent normal mucosa which was selected when available for immunohistochemical staining.

4.1 Total number of patients and exclusions:

A total of 104 patients were included in the study. Three patients who had colorectal cancer diagnosed on colonoscopic biopsy were excluded. Although the original slides showed carcinoma, immunohistochemically stained slides showed tumour tissue insufficient for assessment. A fourth case was excluded because the small biopsy done at laparotomy for bowel obstruction did not include sufficient normal colonic epithelium to serve as positive control. In another 7 cases the tumour, as well as the normal tissue, failed to stain satisfactorily. Staining was repeated with another set of slides but still the result was the same. In the absence of staining of normal colonic epithelium there was no positive control and the results were difficult to interpret. So a total of 93 patients were included in the final analysis.

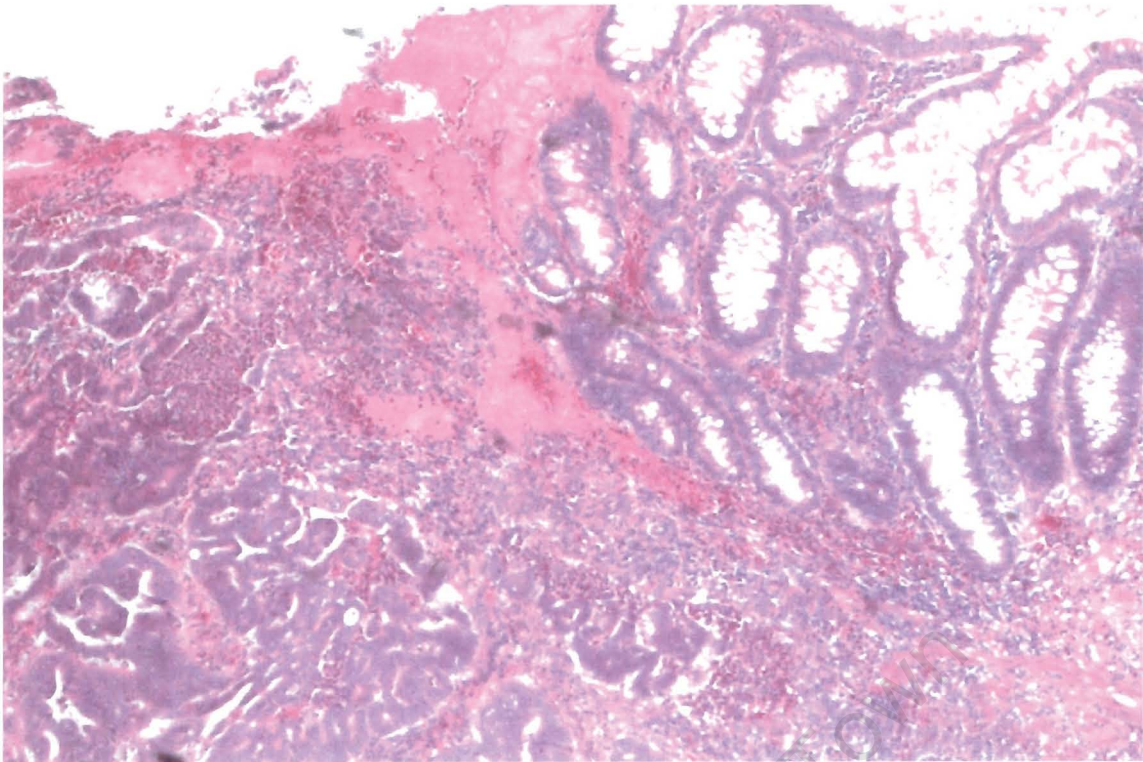


Figure 2: Normal colonic mucosa (right) and moderately differentiated adenocarcinoma of the colon (left). H&E, objective X2.5

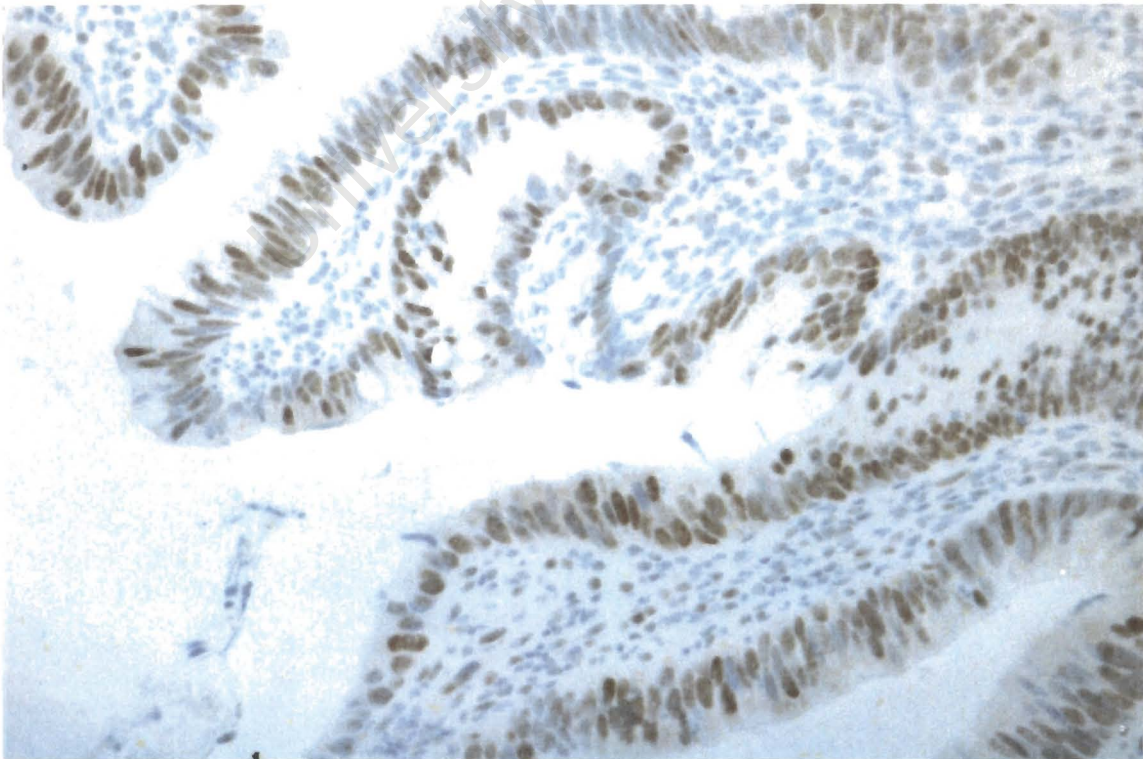


Figure 3: Normal colonic mucosa stained with anti-hMLH1 antibody. Strong positive staining is seen in the nuclei of colonic epithelium. Immunohistochemistry, objective X10

Figure 3 shows normal colonic mucosa stained with anti-hMLH1 antibody. Strong positive staining is seen in the nuclei of colonic epithelium while figure 4 shows adenocarcinoma of the colon from the same patient as normal mucosa in figure 3. Normal colonic mucosa with underlying poorly differentiated carcinoma is seen in Figure 5. Here the nuclei of both normal colonic epithelium and tumour cells show normal expression of hMSH2 protein.

4.2 Frequency of absence of expression of hMLH1 and hMSH2 proteins

Of a total of 93 patients, there were 47 males and 46 females. The mean age was 35 years (range 16-45 years). A total of 56 (60%) patients showed normal expression of both hMLH1 and hMSH2 protein. Twenty-five (27%) patients had absence of expression of hMLH1 protein while 12 (13%) had absence of expression of hMSH2 protein. So a total of 37 (40%) patients showed mismatch repair gene defect due to absent expression of either hMLH1 or hMSH2 protein. Of all patients showing absence of expression of mismatch repair gene protein, 67.5% had *hMLH1* defect while 32.5% had *hMSH2* defect (Table 1). Examples of absence of expression of hMLH1 and hMSH2 proteins in colonic tumour are seen in Figure 6 and 7 respectively. The nuclei of normal colonic epithelium show expression of the protein while adjacent tumour cell nuclei do not show any expression of hMLH1 and hMSH2 protein respectively.

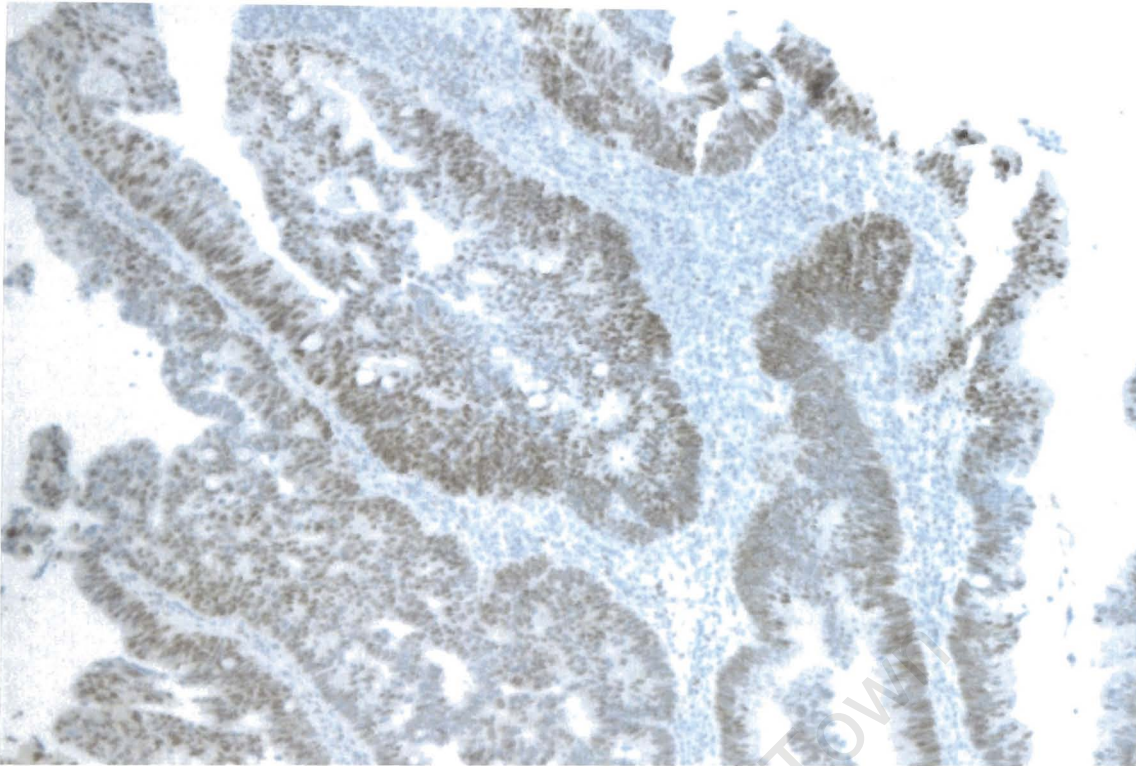


Figure 4: Adenocarcinoma of the colon expressing hMLH1 protein in the nuclei of the tumour cells. Stained with anti-hMLH1 antibody. Immunohistochemistry, objective X4

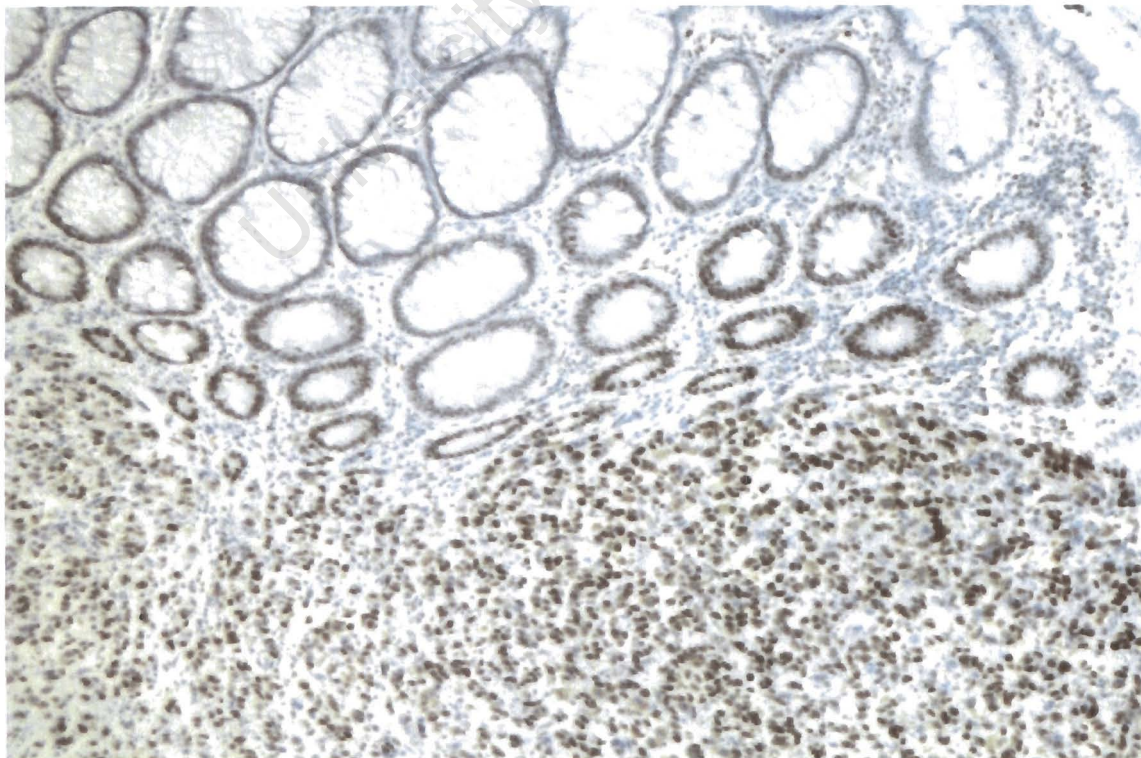


Figure 5: Normal colonic mucosa with underlying poorly differentiated carcinoma. The nuclei in the surface epithelium and tumour cells express hMSH2 protein. Immunohistochemistry, objective X4

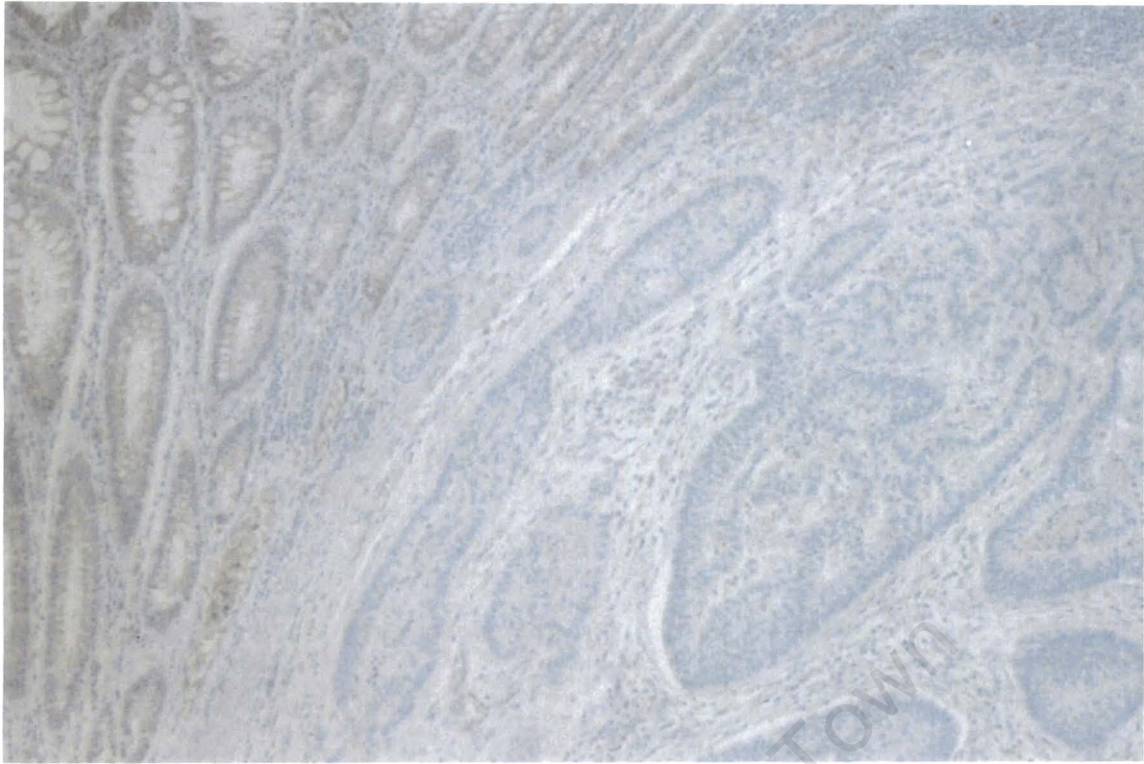


Figure 6: hMLH1 protein expression is seen in the nuclei of the normal colonic epithelium (left). The tumour (right) shows no expression of hMLH1 protein. Immunohistochemistry, objective X4

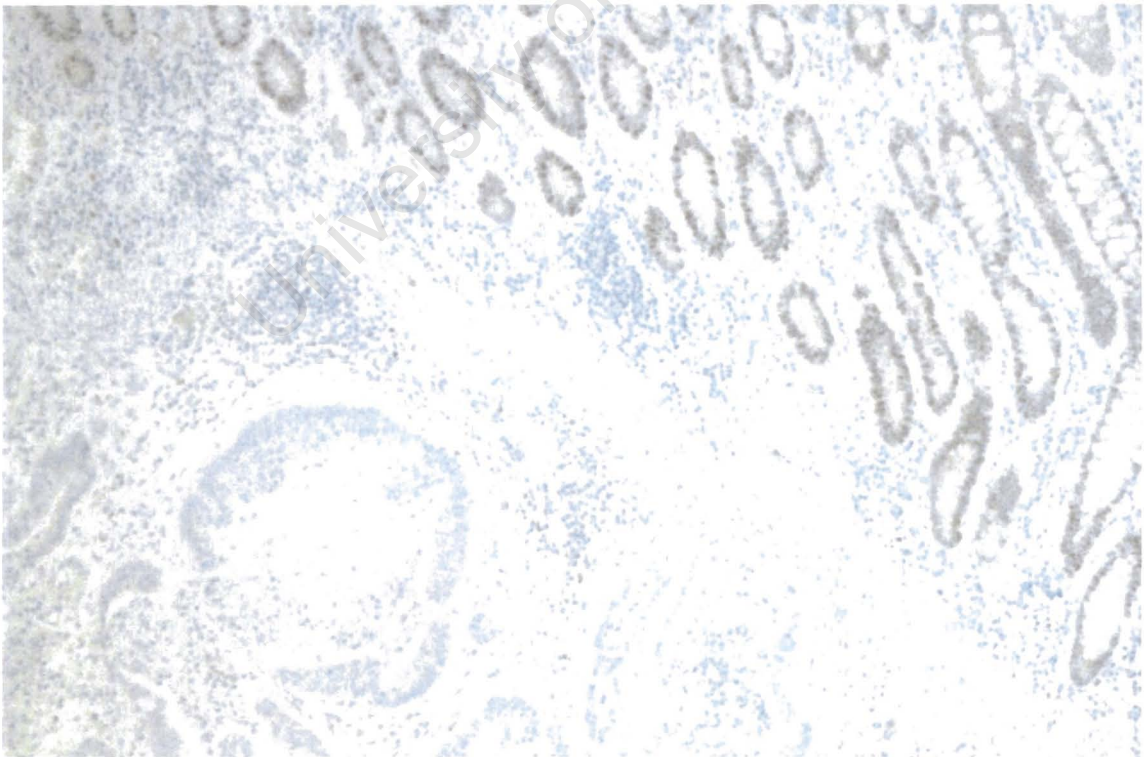


Figure 7: hMSH2 protein expression is seen in the nuclei of the normal colonic epithelium (right). The tumour (left) shows no expression of hMLH1 protein. Immunohistochemistry, objective X4

Table 1: Immunohistochemically detected expression of hMLH1 and hMSH2 proteins in colorectal cancer

<i>No. of patients with expression of hMLH1 and hMSH2</i>	<i>No. of patients with absent expression of hMLH1</i>	<i>No. of patients with absent expression of hMSH2</i>	<i>Total No. of patients with mismatch repair gene defect</i>
n=56	n=25	n=12	37/93
60%	27%	13%	40%

4.3 Clinical and tumour characteristics of patients with and without expression of hMLH1 and hMSH2 proteins

These are shown in Table 2. Age at the diagnosis of carcinoma was similar in the two groups. Male and female ratio was not significantly different in the two groups. One statistically significant finding was the number of right-sided lesions in the MMR deficit group

There were two metachronous lesions in total. One of these was in the group with normal expression of *hMLH1* and *hMSH2* while the other was in the group with absent expression of *hMSH2*. In both patients, 2nd tumour was stained for immunohistochemical analysis.

A total of nine synchronous lesions were found in patients included in this study; four patients showed normal expression of *hMLH1* and *hMSH2* and five did not express either *hMLH1* or *hMSH2*.

Table 2: Clinical and tumour characteristics of patients with intact hMLH1 and hMSH2 protein expression vs. patients with absence of expression of hMLH1 and hMSH2 proteins

<i>Characteristic</i>	<i>Total No. of Patients (n=93)</i>	<i>No. of patients with expression of hMLH1 and hMSH2 (n=56)</i>	<i>No. of patients with absent expression of hMLH1 or hMSH2 (n=37)</i>	<i>p</i>
Age at diagnosis	93	35.1 years	35.3 years	ns
Sex: Male/Female	93	26/30 (46%/54%)	21/16 (56%/44%)	ns
Right sided tumour	40	16 (28%)	24 (69%)	0.0012*
Synchronous lesions	9	4	5	ns
Metachronous lesions	2	1	1	ns

ns = not significant; * = significant result

4.4 Histopathological characteristics of tumours in patients with and without expression of hMLH1 and hMSH2 proteins

These characteristics are shown in table 3. A significantly greater number of poorly differentiated tumours lacked expression of either hMLH1 or hMSH2. A greater number of mucinous tumours also lacked expression of either hMLH1 or hMSH2 proteins. There was no difference in the proportion of Dukes C tumours, or locally advanced tumours in two groups. Figure 8 and 9 highlight the fact that although mucinous tumours are more common in patients with MMR deficit, they do occur in

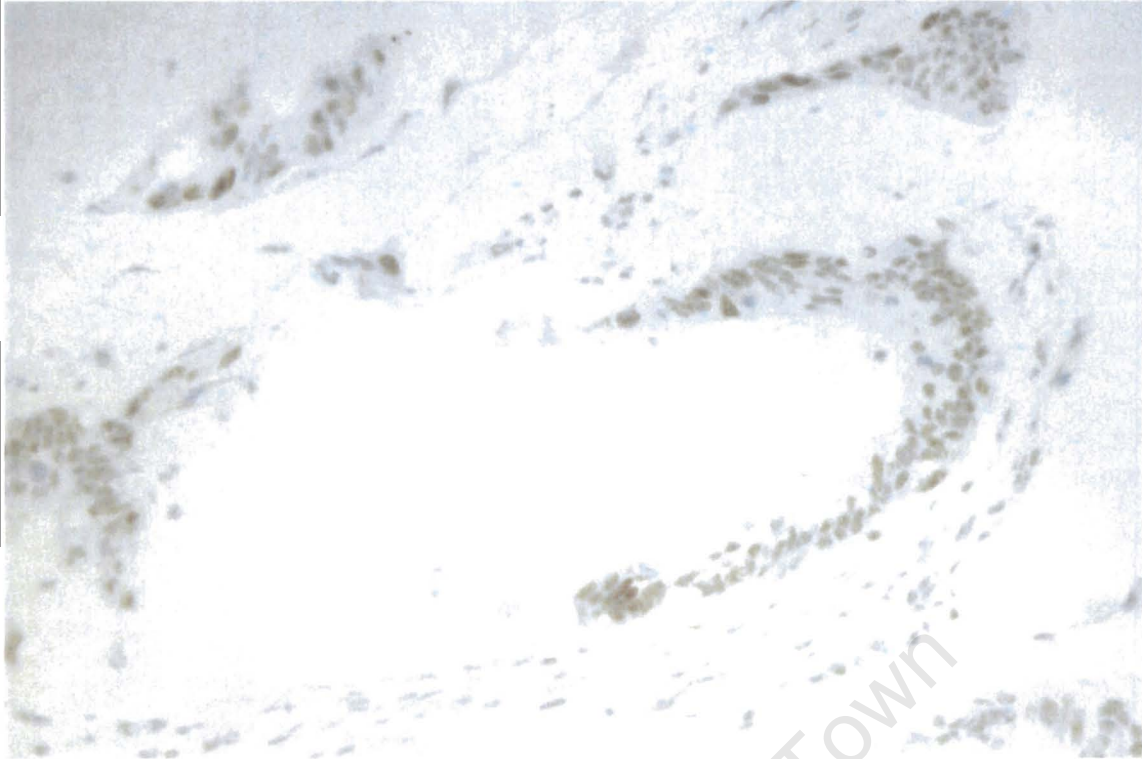


Figure 8: Mucinous adenocarcinoma of the colon composed of large mucin filled glands. The nuclei of the tumour cells express hMLH1 protein. Immunohistochemistry, objective X10

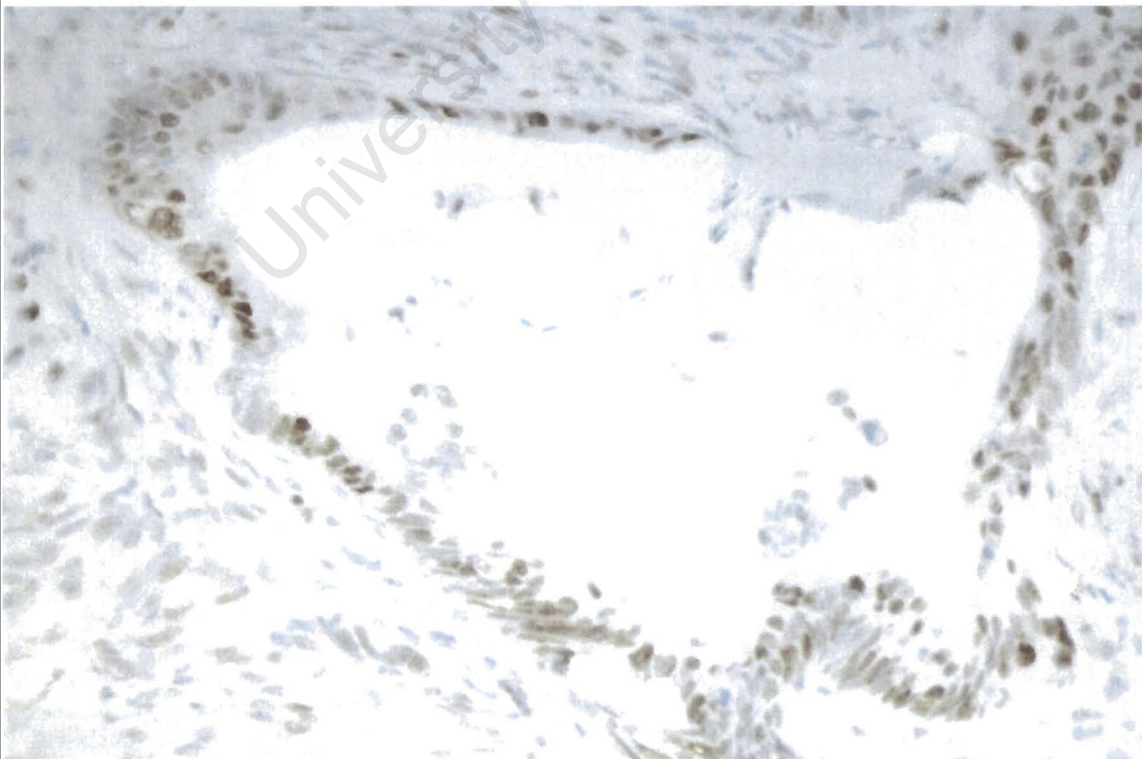


Figure 9: Mucinous adenocarcinoma of the colon composed of large mucin filled glands. The nuclei of the tumour cells express hMSH2 protein. Immunohistochemistry, objective X10

patients who show normal expression of both hMLH1 and hMSH2 proteins. These figures show a mucinous adenocarcinoma of the colon composed of large mucin filled glands. The nuclei of tumour cells express both hMLH1 and hMSH2 proteins respectively.

Table 3. Histopathological characteristics of tumours in patients with and without expression of hMLH1 and hMSH2 proteins

<i>Characteristic</i>	<i>Total No. of Patients[#]</i>	<i>No. of patients with expression of hMLH1 and hMSH2</i>	<i>No. of patients with absent expression of hMLH1 or hMSH2</i>	<i>p</i>
Duke's C Tumours	n=89	31/52 (60%)	23/37 (62%)	ns
Locally Advanced Tumour T ₃ +T ₄ ⁵⁰	n=87	43/52 (83%)	33/35 (94%)	ns
Poor differentiation	n=80	7/48 (15%)	12/32 (38%)	p=0.03 *
>50% mucinous component	n=79	12/50 (24%)	14/29 (48%)	p=0.04 *

ns = not significant; *= significant result; # = number of patients where full data was available

4.5 Survival

Complete follow up information was available for 43 (46%) patients. Censored patients were 50 (54%). These were the patients who were either lost to follow up (they were censored at the date of their last follow up visit) or were still alive at the closure of this study at the end of June 2002. Survival ranged from 40 days to 22.5 years. Figure 10

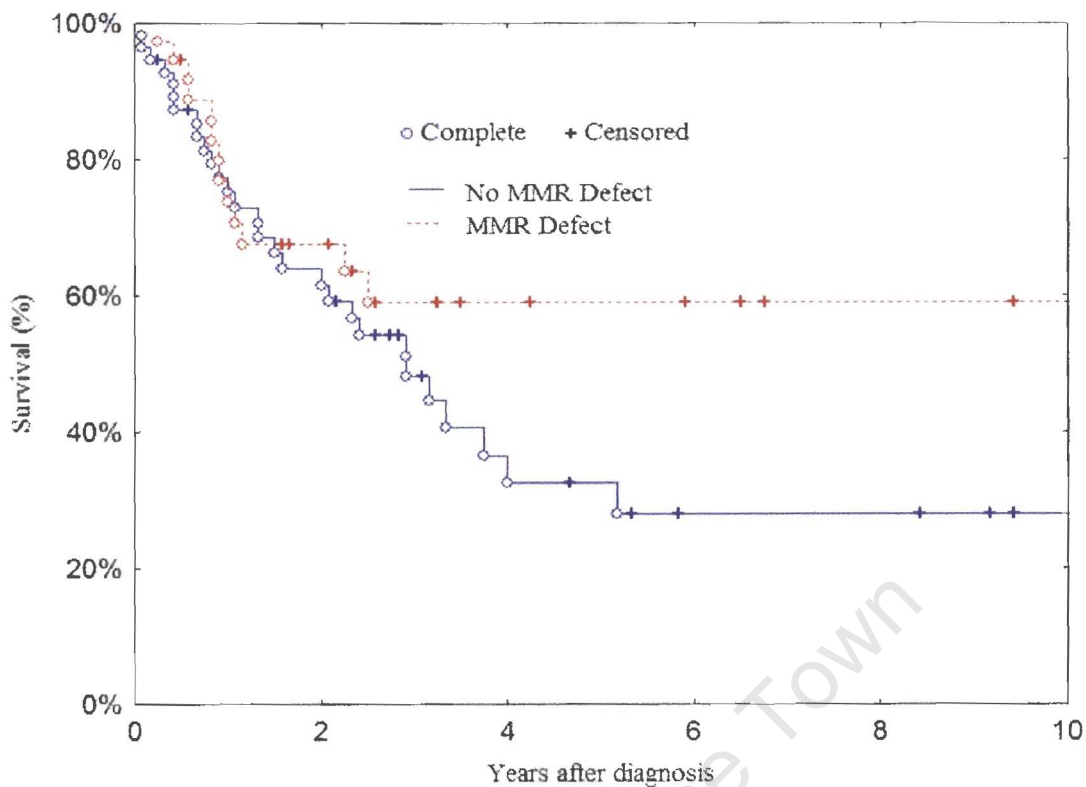


Figure 10: Kaplan-Meier Survival curves for patients with and without mismatch repair defect as determined by immunohistochemistry. Log-rank test: $p=0.16$

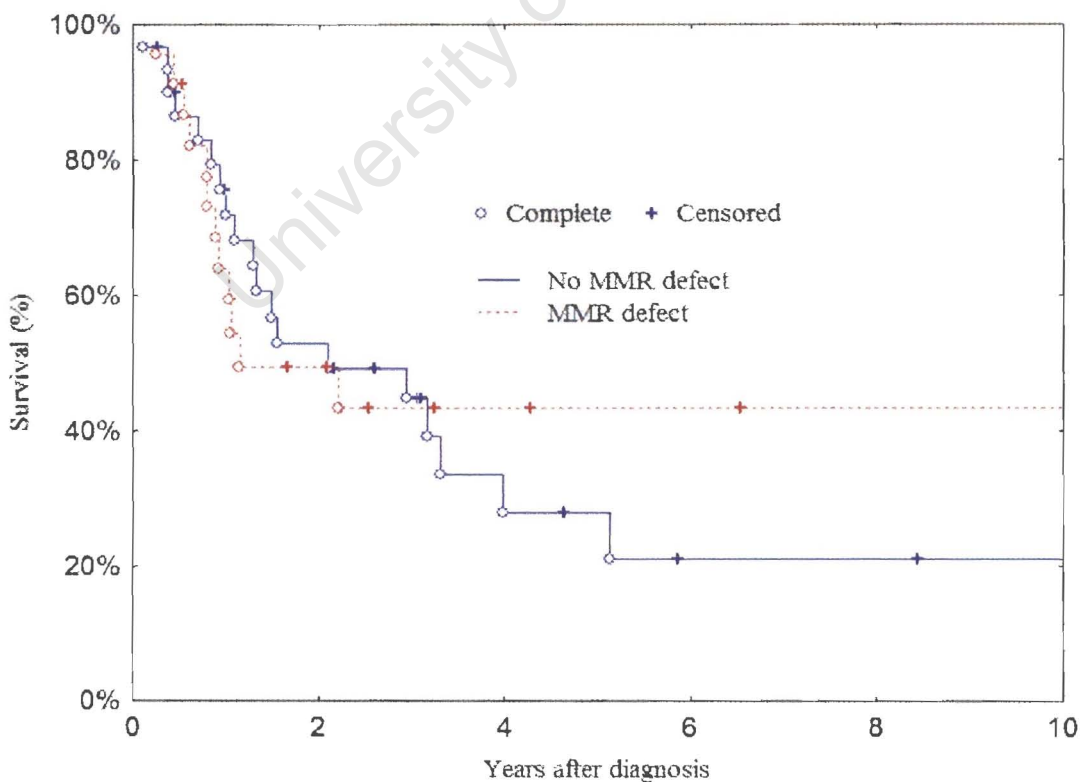


Figure 11: Kaplan-Meier survival curves for Duke's C colorectal cancer patients with and without mismatch repair defect. Log-rank test: $p=0.83$

shows the survival curves for patients with and without MMR defect and Figure 11 shows the survival curves for patients with Duke's C carcinoma stratified by the presence or absence of MMR defect. There was no difference in survival between the two groups.

4.6 Correlation with the mutation status

Immunohistochemical results of patients with colorectal cancer with regard to the presence of MMR defect (absent tumour staining) or its absence (positive tumour staining) were correlated with the mutation status in cases where genetic studies had already been completed by the Human Genetics department of the University of Cape Town. These studies are being done as part of the management of patients with colorectal cancer who are suspected of having hereditary form of the disease. In this study, 56 patients were included from the family cancer programme. These patients had the results of mutation testing as detected by molecular genetic techniques available to me for *hMLH1* and *hMSH2* genes.

Of the 25 patients who had absence of expression of hMLH1 protein implying a defect in the MMR gene *hMLH1*, mutation-testing results were available for 18 patients. Fourteen of these patients were found to carry a disease causing mutation. In four patients no disease causing mutation was identified. Twelve patients in the present study had absence of expression of hMSH2 protein. Genetic mutation testing results for 11 of these patients were available. Three patients were found to have a mutation while in eight patients no mutation was identified by genetic studies. Another important aspect of correlating these results is the finding that out of a total of 56 patients who

had no immunohistochemical evidence of MMR gene defect, 27 had been tested by genetic studies and none of them showed a disease causing mutation. These results are shown in Table 4.

Table 4: Correlation of immunohistochemically determined MMR gene status with genetically determined MMR gene status

<i>MMR gene defect status</i>	<i>Immunohistochemistry (n)*</i>	<i>Genetic mutation identified</i>	
		<i>Yes</i>	<i>No</i>
MMR defect due to <i>hMLH1</i> gene mutation	18	14	4
MMR defect due to <i>hMSH2</i> gene mutation	11	3	8
No MMR gene defect	27	0	27

*= Only patients whose mutation analysis had been done are included in this table

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5. Discussion

It is now widely accepted that most of the colorectal cancers develop through one of the two pathways; chromosomal instability pathway or microsatellite instability pathway.^{16,19,22,60} Cancers developing through these pathways have different phenotypic and biological attributes. There is no readily available and reliable test to differentiate a colorectal cancer arising as a result of chromosomal instability pathway from the one arising due to MMR gene defect. The data from this study suggest that in young patients with colorectal cancer, 40% develop their cancer as a result of a mismatch repair gene defect. Of this group of patients, inactivation of *hMLH1* was responsible for MMR defect in 67% of patients in this study while *hMSH2* inactivation caused MMR defect in the remaining 33%. This MMR gene defect can be detected immunohistochemically by the absence of expression of either *hMLH1* or *hMSH2* protein.

The proportion of patients showing MMR defect due to loss of expression of either *hMLH1* or *hMSH2* gene protein is higher in this study than reported in other studies.^{50,58,61} Gervas et al reported that 24% of their patients with colorectal cancer exhibited high frequency microsatellite instability but the mean age of patients in their study was 70 years.⁶¹ In another study, Gryfe et al found that in young patients ≤ 50 years of age at the diagnosis of colorectal cancer, the proportion of tumours arising by microsatellite instability pathway was 17%.⁵⁰ This percentage was even lower in another study but this also included patients from older age group.⁵⁸ There are several additional explanations for this finding. Colorectal Unit at Groote Schuur Hospital runs a family cancer management programme in collaboration with departments of

Anatomical Pathology and Human Genetics at the University of Cape Town to detect and manage patients with HNPCC. All patients with HNPCC are referred here for management. Surgery for HNPCC mutation carrying patients whose colorectal cancers are diagnosed during surveillance are also managed at this hospital. Because of the design of this study, it was inevitable that some of the young patients who have previously been diagnosed as having HNPCC will be included in this study. However, evaluation of the pathology and immunohistochemically stained slides was done without awareness of diagnosis of familial cancer in these patients. Due to the presence of this cohort of known HNPCC patients in this study, the number of patients identified with mismatch repair defect is higher than normally expected. Moreover, HNPCC frequently presents at a young age so the results of this study do not reflect the frequency of MMR defect in the general population. Groote Schuur Hospital is also an academic referral centre where patients from at least three provinces are referred for multidisciplinary management. This is especially true for patients with colorectal cancer where adjuvant chemoradiotherapy and genetic testing is anticipated. All these factors contributed to a higher proportion of patients with MMR gene defect identified in this study. However some studies have reported an incidence of up to 58% for tumours with MSI due to MMR gene defect if the threshold for patient inclusion is decreased to 35 years of age.^{48,62} Although some of these young patients with colorectal cancers were later shown to have germline mutations in one of the mismatch repair genes, others are true sporadic cancers. In such cases the mechanism of inactivation of MMR gene is hypermethylation of *hMLH1* promoter region.^{3,63} In a prospective study by Ward et al., where consecutive patients undergoing resection of adenocarcinoma of the colorectum were evaluated for MMR gene defect by both microsatellite instability testing and immunohistochemistry, the incidence of MMR

gene defect was 12%. This more likely represents the incidence of colorectal cancer due to mismatch repair gene defect in the general population.⁶⁴

The *hMLH1* was responsible for MMR defect in two-thirds of patients in this study. This finding is in accordance with previous studies showing that mutations in *hMLH1* are responsible for MMR gene defect in majority of patients who develop cancer through MSI pathway.⁶⁵⁻⁶⁸

Among the clinical features and tumour characteristics of the groups with and without MMR defect as detected by immunohistochemistry, the only statistically significant finding was right sided location of the tumours in the MMR gene defect group ($p=0.0012$). This finding confirms previous studies showing that tumours due to microsatellite instability and loss of expression of *hMLH1* or *hMSH2* tend to occur more commonly on the right side.^{3,49,69} In a study of 310 patients analysed for MMR gene defect by MSI testing and immunohistochemistry, 33 tumours were found to have high frequency of microsatellite instability. Twenty six of these 33 tumours were located on the right side while seven were on the left side.⁶⁴ Kim et al., studied 137 colorectal cancer cases and found that in the subgroup with MMR gene defect, the incidence of right sided location of the tumour was 94% while in the group without MMR defect, tumour was located on the right side in 34% of patients.

When histopathological characteristics of the tumours with and without MMR gene defect were compared, it was found that tumours in the groups with MMR gene defect were significantly more likely to be poorly differentiated and mucinous in nature ($p=0.03$ and 0.04 respectively). Although mucinous tumour histology was not found

more commonly in patients with high frequency of MSI in two studies,^{49,50,50} other studies are in accordance with the findings in this study.^{64,69} Considering all these findings, it seems that a significant number of tumours with MMR defect can be suspected by carefully reviewing the location of the tumour and histopathological characteristics of these tumours in young patients with colorectal cancer but the sensitivity of histopathology in predicting the MMR gene defect origin of the cancer is not very high. In one study the sensitivity of histopathology for detection of high frequency of microsatellite instability was 49%.⁷⁰ Tumours with poor differentiation and mucinous tumours do occur in patients without MMR defect (Figure 9 & 10, Table 3) and immunohistochemistry is more sensitive in detecting these tumors.⁷⁰

It has been suggested that survival in patients with colorectal cancer due to MMR gene defect is better than in patients who develop colorectal cancer due to chromosomal instability.^{49,50,55,64} In this study this finding was not statistically significant although Kaplan-Meier survival curves for the two groups show that there is a tendency towards better survival in MMR defect group. This tendency persists even when patients only with Duke's C stage cancer are compared for survival. Again this does not reach statistical significance but this might be due to insufficient number of patients and high proportion of patients who were lost to follow up. If the numbers were large and follow up was more complete, this finding might reach statistical significance. Wright et al. showed that patients with Australian Clinico-Pathologic stage (ACPS) C tumours which displayed high frequency of MSI (origin due to MMR gene defect) had significantly better prognosis than their microsatellite stable counterparts.⁵⁶ The issue of comparison of survival in patients with and without MMR defect is complex due to the use of adjuvant therapy in patients with colorectal cancer without the knowledge of

their mismatch repair defect status and has been the subject of debate in recent studies. In vitro studies have shown that colorectal cancer cell lines with high frequency microsatellite instability are less responsive to fluorouracil than microsatellite stable cell lines.^{51,71,72} However a large study of selected patients and several small series showed improved survival in patients with MMR defect as detected by the presence of high-frequency microsatellite instability.^{52,73,73} But recently a large retrospective study of 570 patients in which 95 patients had high frequency MSI analysed the relationship of high frequency MSI and fluorouracil based chemotherapy to survival. In the 287 patients who did not receive chemotherapy, those displaying high frequency MSI had a better five-year survival than the ones who did not show high frequency MSI. Among the groups of 283 patients who received fluorouracil based adjuvant chemotherapy, high frequency MSI was not associated with improved survival. By multivariate analysis, authors showed that there was no benefit of adjuvant chemotherapy in the group with high frequency MSI.⁵⁵ Other drugs such as the topoisomerase-inhibitor, camptothecin may be more effective in patients with MMR gene defect.⁷⁴ At this stage it seems further prospective studies of adjuvant chemotherapy should stratify patients according to MSI status to clarify these issues. It seems in future, the status of MMR repair gene defect might prove to be one of the most important factors determining the indications and type of chemotherapy used in patients with advanced colorectal cancer.³⁷

When studying the correlation between the immunohistochemically determined MMR defect status and the presence or absence of disease causing mutation in cases where the results of mutation testing by molecular genetic techniques were available, it was clear that immunohistochemistry detected all patients in which *hMLH1* and *hMSH2*

mutation was detected by genetic analysis. But no mutation was detected in 4 patients who did not express hMLH1 protein (*hMLH1* defect) on immunohistochemistry and in 8 patients who did not express hMSH2 protein (*hMSH2* defect) on immunohistochemistry. The explanation for this result is multifactorial. Mutation testing is not 100% accurate; it depends on the technique employed. With the techniques currently used at Human Genetics Department of University of Cape Town, i.e. single stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (dHPLC), it is not possible to detect large genomic deletions in *hMLH1* or *hMSH2*. The knowledge of the number of mutations responsible for MMR defect is not complete. New mutations are added to the database continually as they are being discovered.⁷⁵ There might be mutations on the *hMLH1* and *hMSH2* genes that are yet not known and so are not looked for during genetic analysis of DNA. Converting diploidy to haploidy may also increase the chances of detection of a mutation.⁷⁶ In some cases the cause of gene inactivation may be hypermethylation of the promoter region on *hMLH1* that has not been tested for in these patients.

There were 27 patients in this study whose genetic testing did not show any disease causing mutation on *hMLH1* and *hMSH2*. Four of these patients showed polymorphisms which were though not to be significant because they were present in the controls as well. All of these 27 patients showed normal expression of both hMLH1 and hMSH2 proteins on immunohistochemistry. This is an encouraging finding as it shows that in cases where immunohistochemistry is negative, there is probably no need to look for mutations in *hMLH1* or *hMSH2* genes. Of course if clinical suspicion of cancer arising due to MMR defect is high, mutations should be looked for in other MMR genes like *hMSH6* etc. But these are only minority of cases.⁷

There were 17 patients in this study whose tumours showed variable staining with antibodies on immunohistochemistry. This was more common with anti-hMLH1 staining (12 cases) than with hMSH2 antibody (five cases). Some of these blocks were more than 12 to 15 years old. All of these cases were considered as expressing the hMLH1 and hMSH2 gene protein and included in the group showing no MMR defect. At this moment, it is not clear whether variable immuno-staining is the result of decreased expression of the respective gene product or it is an artefact due to very old specimens. We could not find any technical or biological explanation for this. This problem can probably be resolved by using fresh tissue samples whenever possible. Future MSI testing and genetic mutation analysis will also help clear this issue.

The cost of immunohistochemical testing for hMLH1 and hMSH2 protein expression is 14% to 28% of MSI testing in individuals or families in which the suspicion of HNPCC is high.^{37,77} In our local setting, National Health Laboratory Service requires at least a payment of R200 for doing one immunohistochemistry test. This is not very expensive if other aspects of management of such patients with colorectal cancer are considered. These include specialist radiological investigations like CT scan, mutation analysis, surgery and adjuvant chemo/radiotherapy. Furthermore due to good correlation between immunohistochemistry and results of genetic testing as discussed above, it is probably advisable to first perform immunohistochemistry on patients with colorectal cancer suspected of having a MMR gene defect from clinical features and histopathological characteristics of the tumours. This will also direct the geneticist towards the gene most probably harbouring the mutation indicated by the loss of expression of the gene product. The results of immunohistochemistry can also be used to modify surgical treatment or adjuvant therapy depending on the expression or

absence of expression of the MMR gene protein. For the present time, this will have to be done in the context of clinical trials.

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6. Conclusion

This study demonstrates that a significant proportion (40%) of young patients with colorectal cancer managed at Groote Schuur Hospital/University of Cape Town show absence of expression of either hMLH1 or hMSH2 protein when determined immunohistochemically. Tumours in this subset of patients develop through MMR gene defect pathway. Their tumours are often right sided, poorly differentiated and are mucinous.

This study also shows that there is good correlation between immunohistochemically determined MMR defect status of colorectal cancer patients and results of genetic mutation testing for the presence or absence of MMR defect.

Immunohistochemistry should be considered as a routine initial investigation in young patients diagnosed with colorectal cancer who are suspected of having HNPCC or sporadic colorectal cancer resulting from MMR gene defect. Genetic studies should subsequently be done in patients who have shown MMR gene defect by immunohistochemistry. This could lead to identification of new families with HNPCC syndrome.

References:

1. Ries LAG et al. *SEER Cancer Statistics Review, 1975-2000.*: National Cancer Institute. Bethesda, MD, 2003.
2. South African National Cancer Registry. Internet . 2003.
<http://www.cansa.co.za/registry.asp>
3. Percesepe A et al. Molecular screening for hereditary nonpolyposis colorectal cancer: a prospective population-based study. *Journal of Clinical Oncology* 2001; **19**: 3944-50.
4. Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. *Journal of Medical Genetics* 1999; **36**: 801-18.
5. Aaltonen LA et al. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *The New England Journal of Medicine* 1998; **338**: 1481-7.
6. Moore J, Cowled P. Hereditary non-polyposis colorectal cancer syndrome. *Australian and New Zealand Journal of Medicine* 1999; **69**: 6-13.
7. Lynch HT, de la Chapelle A. Hereditary Colorectal Cancer. *The New England Journal of Medicine* 2003; **348**: 919-32.
8. Nowell PC. The clonal evolution of tumour cell populations. *Science* 1976; **194**: 23-8.

9. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-9.
10. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-70.
11. Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumours. *Science* 1987; **238**: 193-7.
12. Muleris M, Salmon RJ, Zafrani B, Girodet J, Dutrillaux B. Consistent deficiencies of chromosome 18 and of the short arm of chromosome 17 in eleven cases of human large bowel cancer: a possible recessive determinism. *Annales de Genetique (Paris)* 1985; **28**: 206-13.
13. Vogelstein B et al. Genetic alterations during colorectal-tumour development. *The New England Journal of Medicine* 1988; **319**: 525-32.
14. Kern SE et al. Allelic loss in colorectal carcinoma. *Journal of American Medical Association* 1989; **261**: 3099-103.
15. Delattre O et al. Multiple genetic alterations in distal and proximal colorectal cancer. *Lancet* 1989; **ii**: 353-6.
16. Ionov Y, Peinado MA, Mikhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; **363**: 558-61.
17. Aaltonen LA et al. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993; **260**: 812-6.

18. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993; **260**: 816-9.
19. Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000; **119**: 854-65.
20. Groden J et al. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 1991; **66**: 589-600.
21. Leppert M et al. The gene for familial polyposis coli maps to the long arm of chromosome 5. *Science* 1987; **238**: 1411-3.
22. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-67.
23. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398**: 422-6.
24. Peltomäki P et al. Genetic mapping of a locus predisposing to human colorectal cancer. *Science* 1993; **260**: 810-2.
25. Levinson G, Gutman GA. High frequencies of short frameshifts in poly-CA/TG tandem repeats borne by bacteriophage M13 in *Escherichia coli* k-12. *Nucleic Acids Research* 1987; **15**: 5323-38.
26. Strand M, Prolla TA, Liskay RM, Petes TD. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 1993; **365**: 274-6.

27. Fishel R et al. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 1993; **75**: 1027-38.
28. Leach FS et al. Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer. *Cell* 1993; **75**: 1215-25.
29. Bronner CE et al. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* 1994; **368**: 258-61.
30. Papadopoulos N et al. Mutation of a *mutL* Homolog in hereditary colon cancer. *Science* 1994; **263**: 1625-9.
31. Nicolaides NC et al. Mutations of two *PMS* homologues in hereditary nonpolyposis colon cancer. *Nature* 1994; **371**: 75-80.
32. Parsons R et al. Microsatellite instability and mutations of the Transforming Growth Factor β type II receptor gene in colorectal cancer. *Cancer Research* 1995; **55**: 5548-50.
33. Rampino N et al. Somatic frameshift mutations in the *BAX* gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; **275**: 967-9.
34. Markowitz S et al. Inactivation of the type II TGF- β receptor in colon cancer cells with microsatellite instability. *Science* 1995; **268**: 1336-8.
35. Kuismanen SA, Holmberg MT, Salovaara R, de la Chapelle A, Peltomäki P. Genetic and epigenetic modification of *MLH1* accounts for a major share of

- microsatellite-unstable colorectal cancers. *American Journal of Pathology* 2000; **156**: 1773-9.
36. Jass JR et al. Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. *European Journal of Cancer* 2002; **38**: 858-66.
37. Lindor NM et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumours. *Journal of Clinical Oncology* 2002; **20**: 1043-8.
38. Boland CR et al. A National Cancer Institute Workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Research* 1998; **58**: 5246-57.
39. Warthin AS. Heredity with reference to carcinoma. *Archives of Internal Medicine* 1913; **12**: 546-55.
40. Lynch HT, Krush AJ. Cancer family "G" revisited: 1895-1970. *Cancer* 1971; **27**: 1505-11.
41. Liu B et al. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Medicine* 1996; **2**: 169-74.
42. Watson P, Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. *Cancer* 1993; **71**: 677-85.

43. Vasen HFA et al. The risk of brain tumours in hereditary nonpolyposis colorectal cancer. *International Journal of Cancer* 1996; **65**: 422-5.
44. Sijmons RH, Kiemeneij LA, Witjes JA, Vasen HFA. Urinary tract cancer and hereditary nonpolyposis colorectal cancer: risks and screening options. *Journal of Urology* 1998; **160**: 466-70.
45. Vasen HFA, Watson P, Mecklin J, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the international collaborative group on HNPCC. *Gastroenterology* 1999; **116**: 1453-6.
46. Vasen HFA, Mecklin J-P, Meera Khan P, Lynch HT. The international collaborative group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). *Diseases of Colon and Rectum* 1991; **34**: 424-5.
47. Goldberg PA et al. In a resource-poor country, mutation identification has the potential to reduce the cost of family management for hereditary nonpolyposis colorectal cancer. *Diseases of Colon and Rectum* 2001; **41**: 009-10.
48. Liu B, Farrington SM, Petersen GM. Genetic instability occurs in the majority of young patients with colorectal cancer. *Nature Medicine* 1995; **1**: 348-52.
49. Lukish JR et al. Prognostic significance of DNA replication errors in young patients with colorectal cancer. *Annals of Surgery* 1998; **227**: 51-6.
50. Gryfe R et al. Tumour microsatellite instability and clinical outcome in young patients with colorectal cancer. *The New England Journal of Medicine* 2000; **342**: 69-77.

60. Sutherland F, Haine L, Quirke P. Molecular approaches to colorectal cancer. *Current Diagnosis in Pathology* 1998; 5: 34-43.
61. Gervaz P et al. Comparison of microsatellite instability and chromosomal instability in predicting survival of patients with T3N0 colorectal cancer. *Surgery* 2002; 131: 190-7.
62. Datta RV, LaQuaglia MP, Paty PB. Genetic and phenotypic correlates of colorectal cancer in young patients. *The New England Journal of Medicine* 2000; 342: 137-8.
63. Herman JG et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proceedings of National Academy of Sciences of USA* 1998; 95: 6870-5.
64. Ward R et al. Microsatellite instability and the clinicopathologic features of sporadic colorectal cancer. *Gut* 2001; 48: 821-9.
65. Marcus VA et al. Immunohistochemistry for hMLH1 and hMSH2: a practical test for DNA mismatch repair-deficient tumours. *American Journal of Surgical Pathology* 1999; 23: 1248-55.
66. Thibodeau SN et al. Altered expression of hMSH2 and hMLH1 in tumours with microsatellite instability and genetic alterations in mismatch repair genes. *Cancer Research* 1996; 56: 4836-40.

67. Thibodeau SN et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Research* 1998; **58**: 1713-8.
68. Chaves P et al. Immunohistochemical detection of mismatch repair gene protein as a useful tool for the identification of colorectal carcinoma with the mutator phenotype. *Journal of Pathology* 2000; **191**: 355-60.
69. Kim H, Jen J, Vogelstein B, Hamilton SR. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *American Journal of Pathology* 1994; **145**: 148-56.
70. Alexander J et al. Histopathological identification of colon cancer with microsatellite instability. *American Journal of Pathology* 2001; **158**: 527-35.
71. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. *Clinical Cancer Research* 1998; **4**: 1-6.
72. Branch P, Aquilina G, Bignami M, Karran P. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature* 1993; **362**: 652-4.
73. Hemminki A, Mecklin J-P, Järvinen H, Aaltonen LA, Joensuu H. Microsatellite instability is a favorable prognostic indicator in patients with colorectal cancer receiving chemotherapy. *Gastroenterology* 2000; **119**: 921-8.

74. Bras-Goncalves RA et al. Sensitivity to CPT-11 of xenografted human colorectal cancers as a function of microsatellite instability and p53 status. *British Journal of Cancer* 2000; 82: 913-23.
75. International Collaborative Group on HNPCC. World Wide Web (Internet) . 2003.
76. Yan H et al. Conversion of diploidy to haploidy. *Nature* 2000; 403: 723-4.
77. Debniak T, Kurzawski G, Gorski Bea. Value of pedigree, clinical data, immunohistochemistry and microsatellite instability analyses in reducing the the cost of determining *hMLH1* and *hMSH2* gene mutations in patients with colorectal cancer. *European Journal of Cancer* 2000; 36: 49-54.

Appendix A

The use of Immunohistochemical detection of mismatch repair deficit in patients with colorectal cancer

MF Hameed¹, P Hall², R Barnard², U Algar¹, R Felix³, RS Ramesar³, PA Goldberg¹,
Department of Surgery¹, Anatomical Pathology², and Human Genetics³, Groote
Schoor Hospital and University of Cape Town, South Africa

Mutations in mismatch repair genes are responsible for the majority of colorectal cancers in patients with Hereditary Nonpolyposis Colorectal Cancer. Only a minority of apparently sporadic cases have defects of mismatch repair. Until recently the only way to detect mismatch repair deficit was by polymerase chain reaction analysis. This is a highly specialized, expensive and time-consuming investigation.

Aim: To test a recently described immunohistochemical technique for the detection of mismatch repair gene protein

Methods: Paraffin sections of the colorectal cancers in 10 patients (females :4, males: 6, mean age at diagnosis 35.8 years) known to have inherited hMLH1 mutation and 10 patients (Females: 6, males: 4, mean age at diagnosis: 71.7 years) thought to have sporadic cancers were studied. Sporadic cancers were defined as cancers occurring in patients with no family history of colorectal cancer and age more than 60 years at the time of diagnosis. These sections were dewaxed and rehydrated. Endogenous peroxidase was blocked using 2% hydrogen peroxide. Antigenic sites were retrieved using Antigen Unmasking Solution. The monoclonal hMLH1 antibody (PharMingen, San Diego, California, USA) was applied in a final dilution of 1/70 in a solution of

0.2x casein in Tris buffered saline (pH 7.6) at room temperature for 2 hours. A StreptABComplex/HRP Duet Kit (Dako, UK) was applied. The reactions were visualized using 0.02% diaminobenzidine in Tris buffered saline (pH 7.6). The sections were counterstained with haematoxylin. All slides were scored by two investigators who were unaware of the clinical features and mutation status of the patients studied. The presence of mutation in the genes was shown by absence of staining of tumour cell nuclei by the antibody, implying the absence of normal protein product of mismatch repair gene. The nuclei of the adjacent normal cells stained.

Results:

	n	<u>hMLH1 Gene Product Staining</u>		
		-	+	-/+
Sporadic	10	1	9	-
HNPCC	10	7	-	3

Conclusion:

The immunohistochemical technique to detect mismatch repair deficit works. It is quick and relatively inexpensive. It may be useful as an initial screening technique for patients with colorectal cancer suspected of Hereditary Non-polyposis Colorectal Cancer Syndrome.

Appendix B

The role of immunohistochemistry in the identification of mismatch repair gene deficit in young patients with colorectal cancer

MF Hameed¹, P de la M Hall², R Barnard², RS Ramesar³, PA Goldberg¹.

Department of Surgery¹, Anatomical Pathology², and Human Genetics³, Grootte Schuur Hospital and University of Cape Town

Background and Objective: An immunohistochemical technique for the detection of mismatch repair gene (hMLH1) protein has been validated in our laboratory using tumours from genetically proven HNPCCs; in this study we used this technique to detect mismatch repair deficit in young patients with colorectal cancers.

Methods: Colorectal cancers in 46 patients 45 years of age or younger were studied. Paraffin sections of tumor and adjacent normal colonic tissue were dewaxed and hydrated. After unmasking antigenic sites, the monoclonal hMLH1 and hMSH2 antibodies were applied in a 1/70 solution of 0.2x casein in Tris buffered saline (pH 7.6) at room temperature for 2 hours. Two investigators separately assessed the results in coded slides. The presence of mutation in the genes was shown by absence of staining in tumor cell nuclei, implying the absence of normal protein product of mismatch repair gene.

Results: 12/46 tumours were negative for hMLH1 and 5/46 negative for hMSH2 indicating abnormal MMR gene protein. All seven patients with proven HNPCC were identified successfully by this technique.

Conclusions:

1. MMR gene deficit in colon cancers can be detected quickly and relatively inexpensively by immunohistochemistry.
2. This cost-effective method could be used for the initial screening of young patients with colorectal cancer; however mutation detection will still be required to confirm or exclude HNPCC.

University of Cape Town